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Specific *in vivo* binding to the norepinephrine transporter demonstrated with the PET radioligand, (*S,S*)-[^{11}C]MeNER

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Abstract

(*S,S*)-2-(α-(2-Methoxyphenoxy)benzyl)morpholine (MeNER), an *O*-methyl analog of the selective and potent norepinephrine transporter (NET) inhibitor, (*S,S*)-reboxetine, and its less active enantiomer, (*R,R*)-MeNER, have each been radiolabeled by *O*-methylation of their corresponding phenolic precursors in good yields from [^{11}C]methyl iodide or [^{11}C]methyl triflate. Radiochemical purities were >99% and specific radioactivity at time of injection was about 74 GBq/μmol. Autoradiographic examination of (*S,S*)-[^{11}C]MeNER binding to human brain slices post mortem indicated specific binding in a brain region including the locus coeruleus. PET examination of both [^{11}C]MeNER enantiomers in a cynomolgus monkey demonstrated a higher specific binding of the (*S,S*)–enantiomer with ratios of 1.4-1.6 in the lower brainstem, mesencephalon and thalamus to striatum. Pretreatment with the NET ligand, desipramine, decreased the specific binding of (*S,S*)-[^{11}C]MeNER. Labeled metabolites of [^{11}C]MeNER were all more polar. (*S,S*)-[^{11}C]MeNER is a good lead compound in the search for a selective radioligand for quantitation of NET in the human brain *in vivo*.

Keywords: Norepinephrine transporter; PET; Monkey; Autoradiography; MeNER

1. Introduction

The neurotransmitter, norepinephrine (NE), and its reuptake system have long been of central interest in relation to the pathophysiology and treatment of neuropsychiatric disorders. The norepinephrine transporter (NET) is the membrane glycoprotein responsible for reuptake of NE from the synapse [1,6] and is, like the serotonin transporter (SERT), a recognized target for antidepressant drug treatment. The locus coeruleus is a brain structure known to be rich in NET [19], and implicated in current hypothesis on the pathophysiology and treatment of depression [15]. High densities of NETs also exist in the hypothalamus and thalamus whereas low densities have been demonstrated in the cerebellum and striatum [2,3,5,8,15,19,24]. *In vivo* brain imaging of NET with positron emission tomography (PET) or single photon emission tomography (SPET) would offer a tool to gain knowledge about mechanisms regarding antidepressant drug treatment, that may lead to more efficient clinical practice. For the two other major monoamine transporters selective PET and SPET radioligands have already been developed. These include [^{11}C]PE2I and [^{123}I]β-CIT for the dopamine transporter (DAT) [12,18] and [^{11}C]MADAM, [^{11}C]DASB and [^{123}I]ADAM for SERT [20,25,30]. [^{3}H]Nisoxetine (**3,** Fig. 1) has been used for *in vitro* autoradiographic studies of NETs [3,15,24] but about half of the binding of [^{11}C]nisoxetine in mice (50-65%) *in vivo* was found to be non-specific [9]. The preparation of [^{11}C]desipramine has been reported [27] but preliminary *in vivo* data obtained with PET indicated that the degree of specific binding in the monkey brain was too low to allow for visualization of NET [23]. An iodo-derivative of tomoxetine has also been prepared as a potential SPET ligand [4,7], but *in vivo* data has not been reported up to date. (*S,S*)-MeNER (**1,** Fig. 1) is an *O*-methyl analog of reboxetine (**2,** Fig. 1). Reboxetine is marketed as a mixture of (*R,R*) and (*S,S*) enantiomers, of which the (*S,S*)-enantiomer has been shown to be more than 20 times more potent [32]. The *in vitro* affinity of MeNER for NET is 2.5 nM (IC_{50}) [16], about three times more potent than that of reboxetine (IC_{50} = 8.23 nM) [16]. MeNER is selective, about 100-fold

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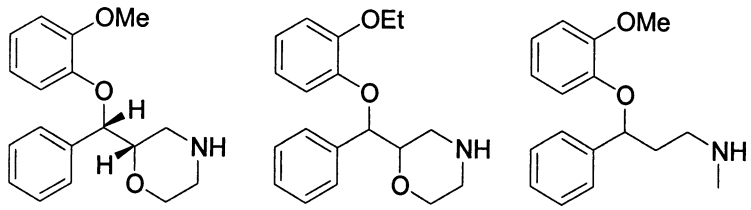

1 2 3

Fig. 1. Structures of (*S.S*)-MeNER (**1**), reboxetine (**2**) and nisoxetine (**3**).

over SERT and >4000-fold over DAT [16], less lipophilic than reboxetine (clog P = 2.91 vs 3.52 [17]) and altogether possesses promising physicochemical and pharmacological properties for a suitable PET radioligand. The aims of the present study were to i) prepare each of the enantiomers of [^{11}C]MeNER, ii) examine [^{11}C]MeNER binding *in vitro* by autoradiographic analysis of post-mortem human whole hemisphere cryosections exposed to [^{11}C]**1** and iii) perform preliminary PET examinations in cynomolgus monkeys, including radioligand metabolism measurements with gradient HPLC.

2. Materials and methods

2.1. Radiochemistry

2.1.1. General procedures

N,N-Dimethylformamide (DMF) was obtained from Merck, distilled under vacuum and dried over molecular sieves (4Å). The precursors and standards (enantiomers of MeNER) were supplied by Eli Lilly, Indianapolis, US. Other chemicals were obtained from commercial sources and were of analytical grade. [^{11}C]Carbon dioxide was produced at the Karolinska Hospital with a GEMS PETtrace cyclotron using 16.4 MeV protons in the ^{14}N(p,α)^{11}C reaction on nitrogen gas. [^{11}C]Methyl iodide was synthesized from [^{11}C]carbon dioxide utilizing a GEMS PETtrace MeI Microlab® system [22]. [^{11}C]Methyl triflate was prepared by sweeping [^{11}C]methyl iodide vapor through a heated glass column containing silver-triflate-impregnated graphitized carbon, as previously described [22]. Semi-preparative reversed-phase HPLC was performed using a Waters μ-Bondapak C-18 column (300 x 7.8 mm, 10 μm) and an absorbance detector (λ = 254 nm) in series with a GM-tube for radiation detection. [^{11}C]MeNER was purified by HPLC using system A (CH_3CN-NH_4CO_2H (0.1 M); 30:70 v/v) as mobile phase at 6 mL/min. The radiochemical purity of each product was determined by reversed phase HPLC equipped with a Waters μ-Bondapak C-18 column (300 x 3.9 mm, 10 μm) and an absorbance detector (λ = 254 nm) in series with a Beckman β-flow radiodetector for radiation detection on mobile phase system B (CH_3CN-H_3PO_4 (10 mM), 30:70 v/v) at 2 mL/min. [^{11}C]MeNER was identified by co-injection with unlabeled MeNER.

*2.1.2. (S,S)-[^{11}C]MeNER (**1**, Fig. 2)*

[^{11}C]Methyl iodide was trapped at room temperature in a reaction vessel containing 1.0 mg of the phenolic precursor **4,** DMF (400 μL) and sodium hydroxide (5 M, 6 μL). The vessel was sealed and heated at 90°C for 3 min. Mobile phase (System A, 600 μL) was added to the crude reaction mixture before its injection onto the semi-preparative HPLC column (t_R 8-9 min, system A, flow 6 mL/min, Fig. 3). After evaporation of mobile phase, the residue was dissolved in sterile disodium phosphate buffered saline (pH 7.4; 8 mL) and filtered through a sterile Millipore filter (0.22 μm), yielding [^{11}C]**1** in a sterile solution free from pyrogens.

*2.1.3. (R,R)-[^{11}C]MeNER (**6**, Fig. 2)*

[^{11}C]Methyl triflate was trapped at room temperature in a reaction vessel containing 0.5 mg of the Boc-protected phenolic precursor **5,** acetone (400 μL) and sodium hydroxide (0.5M, 2 μL). TFA (90 μL) was then added and the resulting mixture was heated to 90 °C for 4 min.

2.2. In vitro autoradiography

The human brain used was obtained from the National Institute of Forensic Medicine, Karolinska Institutet, Stockholm, Sweden. The brain had been removed at clinical autopsy and was handled in a manner similar to that described earlier [10]. The sections were incubated for 20 min at room temperature with [^{11}C]**1** in a 50 m M TRIS buffer, pH 7.4, containing 300 mM sodium chloride, 5 m M potassium chloride and 0.1% (w/v) ascorbic acid. The sections were then washed (same buffer) 3 times and briefly dipped in cold distilled water before being exposed to Kodak Biomax MR film overnight. Non-specific binding was studied by simultaneous incubation with the selective NET inhibitor, maprotiline (10 μM).

2.3. Positron emission tomography

The Siemens ECAT EXACT HR PET system was run in 3D mode. The spatial resolution is about 3.8 mm FWHM. Images were displayed as 47 sections with a separation of 3.3 [28]. Two cynomolgus monkeys (5060 and 5975 g) were supplied by the National Institute for Infectious Disease Control, Solna, Stockholm. The study was approved by

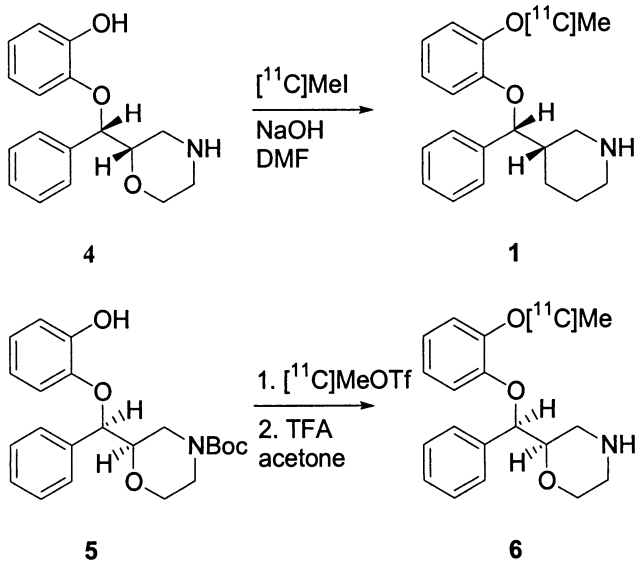

Fig. 2. Preparation of both enantiomers of [^{11}C]MeNER.

the Animal Ethics Committee of Northern Stockholm. Anesthesia was induced and maintained by repeated intramuscular injection of a mixture of ketamine (3-4 mgkg^{-1}h^{-1} Ketalar®, Parke-Davis) and Xylazine hydrochloride (1-2 mgkg^{-1}h^{-1} Rompun® vet., Bayer Sweden). A fixation system was used to secure a position of the monkey head during the PET experiments [14]. Body temperature was controlled by a heating pad with thermostat. In each PET experiment 50-59 MBq of [^{11}C]MeNER (*S,S* or *R,R*) was injected as a bolus into a sural vein. Radioactivity in brain was measured according to a pre-programmed sequence of frames during 93 min. In the first monkey, two baseline experiments were performed (both enantiomers). In the second monkey a baseline experiment with [^{11}C]**1** was performed followed by a pretreatment experiment in which the selective NET inhibitor, desipramine (DMI; 5 mg/kg), was injected intravenously 20 min before injection of [^{11}C]**1.** Both experiments were performed on the same day.

2.3.1. Regions of interest

Regions of interest (ROI's) (lower brainstem, mesencephalon, striatum and thalamus) were drawn on the summation images which were reconstructed from 9 to 93 min after injection of [^{11}C]MeNER and were defined on each section according to the monkey version of the Human Brain Atlas system (HBA) developed at the Karolinska Institutet [21]. Radioactivity was calculated for the se-

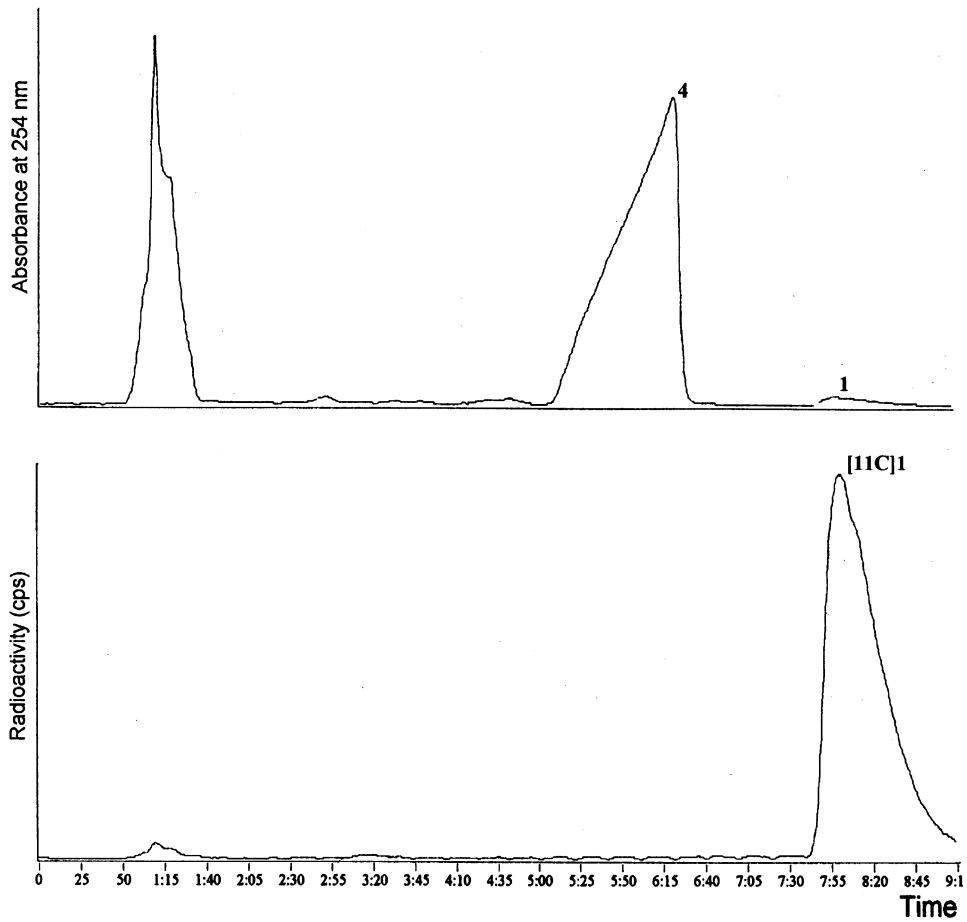

Fig. 3. Preparative HPLC chromatogram from the purification of [^{11}C]**1**.

quence of time frames, corrected for the radioactivity decay and plotted versus time. The percent of injected [^{11}C]MeNER present in brain at time of maximal radioactivity concentration, (i.e. 18 min after the injection of [^{11}C]MeNER) was used as an index of drug uptake in the brain. This percentage was calculated by multiplying the brain volume with the radioactivity concentration in the ROI for the whole brain divided by the injected dose of radioactivity. The brain volume was calculated by multiplying the sum of the whole brain regions of all PET-sections with the plane separation. Striatum, which contains very low densities of NETs, was used as a reference region for the free radioligand concentration and non-specific binding. To calculate specific binding, radioactivity in the striatum was subtracted from the radioactivity in a ROI.

2.4. Plasma metabolite studies

The method for determination of the percentages of radioactivity corresponding to unchanged [^{11}C]MeNER in monkey plasma and labeled metabolites during the time of a PET experiment was a modification of an HPLC method that has been demonstrated to be useful for other PET radioligands [11]. Blood samples (2 mL) were obtained venously from the monkey at 4, 15, 30 and 45 min after injection of [^{11}C]MeNER. Plasma (0.5 mL) obtained after centrifugation at 2000 g for 2 min was mixed with acetonitrile (0.7 mL). The supernatant acetonitrile-plasma mixture (1.1 mL) and the precipitate obtained after centrifugation at 2000 g for 2 min were counted in a NaI well-counter. The HPLC system used in the experiments consists of a Hitachi D-7000 interface module, a Hitachi L-7100 pump, a Rheodyne injector (7125 with a 1.0 mL loop) equipped with a Waters μ-Bondapak-C18 column (300 x 7.8 mm, 10 μm) and a Hitachi L-7400 absorbance detector (254 nm) in series with a Packard Radiomatic 150TR radiodetector equipped with a PET Flow Cell (600 μL cell). Phosphoric acid (10 mM) (C) and acetonitrile (D) were used as mobile phase at 6.0 mL/min. HPLC program; 0-5.5 min, (C/D) 90/10-40/60; 5.5-6.5 min, (C/D) 40/60-90/10; 6.5-10 min (C/D) 90/10 isocratic. The radioactive peak having a retention time corresponding to standard MeNER was integrated and its area was expressed as a percentage of the sum of the areas of all detected radioactive peaks.

3. Results

3.1. Radiochemistry

The incorporation yield of [^{1}C]methyl iodide or [^{11}C]methyl triflate into [^{11}C]MeNER was quantitative using both pathways (Fig. 2). The total synthesis time was 30-35 min and the radiochemical purity was better than 99% (t_R 4-5 min, system B, flow 2 mL/min). The specific radioactivity at time of injection was about 74 GBq/μmol (2000 Ci/mmol) corresponding to an injected dose of about 0.2 μg MeNER.

3.2. In vitro autoradiography

[^{11}C]**1** bound to gray matter in cerebral cortical areas. Binding could also be demonstrated in the brainstem in a region that could correspond to the locus coeruleus. The addition of a high concentration (10 μM) of maprotiline inhibited part of the [^{11}C]**1** binding in the cortical area as well as in the locus coeruleus (Fig. 4).

3.3. Positron emission tomography

The time- radioactivity curves after baseline intravenous injections of the two [^{11}C]MeNER enantiomers demonstrated a higher specific binding for the (*S,S*)-enantiomer than for the (*R,R*)-enantiomer (Fig. 5A). At 18 min after intravenous baseline injection of [^{11}C]MeNER approximately 3% of the total radioactivity was present in the monkey brain. The highest uptake of radioactivity was found in the lower brainstem, mesencephalon and thalamus whereas radioactivity was lowest in striatum (Fig. 5B, 6). Specific binding in lower brainstem, mesencephalon and thalamus increased during the whole experiment (Fig. 5C). Higher uptake to brain was obtained in the pre-treatment experiment (3.5%) compared to the baseline experiment. In the pretreatment experiment, DMI inhibited [^{11}C]**1** binding to almost the same level as in striatum, resulting in a decrease of specific binding (Fig. 5D).

3.4. Plasma metabolite studies

The injected radioactivity eluted in HPLC within 7 min with a good resolution of unchanged radioligand from the labeled metabolites (Fig. 7). The amount of the total radioactivity in plasma representing unchanged [^{11}C]**1** was 85-90% at 18 min and 70-80% at 45 min.

4. Discussion

4.1. Radiochemistry

Two unidentified radioactive products were formed when reacting **4** with [^{11}C]methyl triflate at low concentrations of sodium hydroxide, of which one probably corresponds to *N*-[^{11}C]methylated **4.** Alternatively, methylation of the Boc-protected precursor **5** with [^{11}C]methyl triflate proceeded well, but required a second deprotection step with TFA. Instead, by using an excess of sodium hydroxide in the reaction of the phenolic precursor **4** with [^{11}C]methyl iodide, no significant *N*-methylation of the morpholino nitrogen was observed. Accordingly, due to the simplicity of this one step procedure, we decided to use it as the labeling method of choice for MeNER. Mobile phase system B was

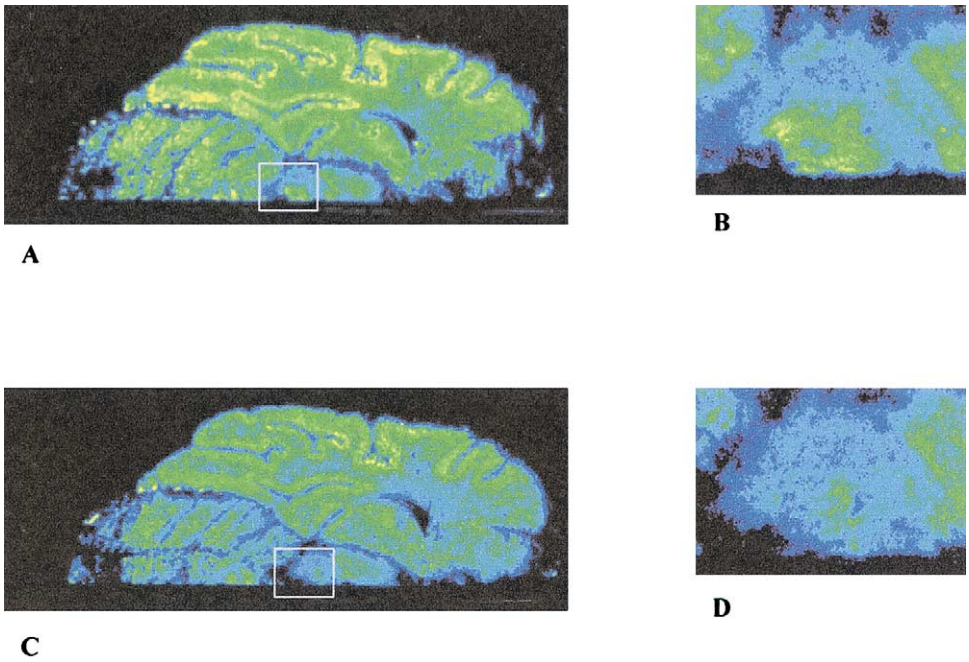

Fig. 4. Color-coded autoradiograms of human whole hemisphere cryosections labeled with [^{11}C]**1**, A, B) 40 MBq [^{11}C]**1**, C, D) 40 MBq [^{11}C]**1** + 10 μM maprotiline. (Brainstem enlarged in images B and D).

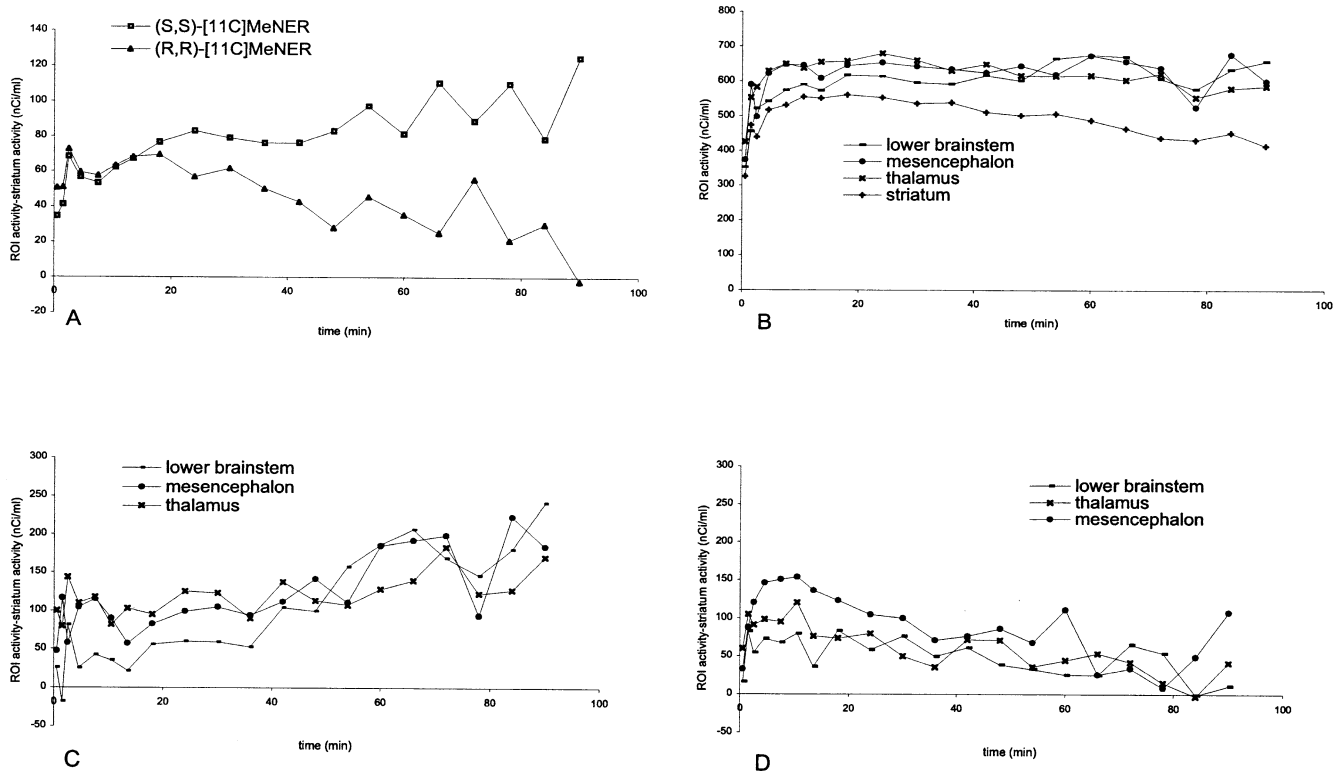

Fig. 5. Time-radioactivity curves derived from PET experiments with [^{11}C] MeNER in cynomolgus monkey. A) Specific binding in the mesencephalon following baseline i.v. injection of [^{11}C]**1** and [^{11}C] **6**. B) [^{11}C]**1** distribution in brain regions. C) Specific binding following baseline injection of [^{11}C]**1**. D) Specific [^{11}C]**1** binding in the pretreatment experiment.

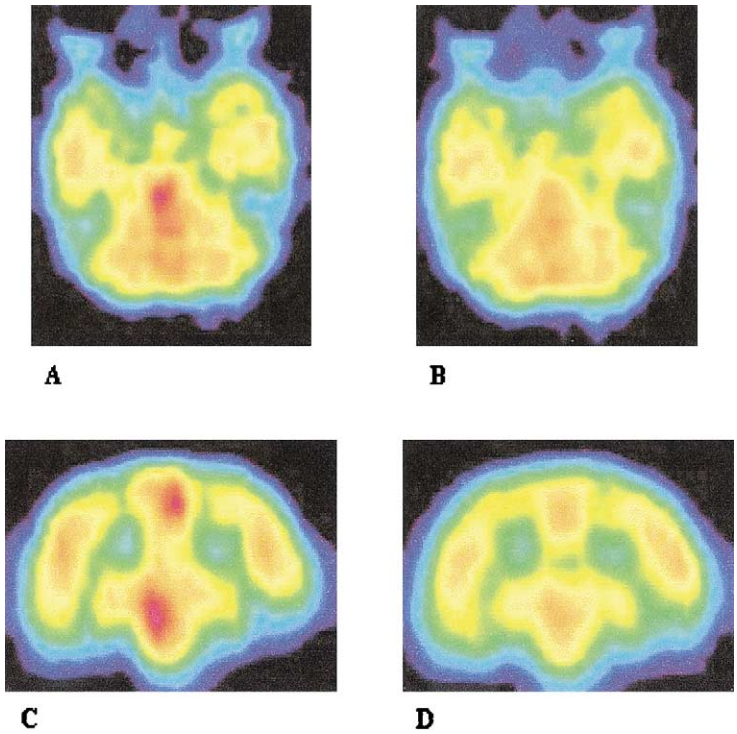

Fig. 6. Color coded PET images showing the distribution of radioactivity in a cynomolgus monkey brain at baseline (A, C) and pretreatment (B, D) experiments with [^{11}C]**1**. A, B) Horizontal images at the level of mesencephalon. (C, D) Coronal images at the level of mesencephalon and pons.

initially used in preparative purification attempts, but since a large amount of radiolysis was observed after evaporation (up to 40%), the mobile phase was changed from system B to system A.

4.2. In vitro autoradiography

The autoradiograms showed that (*S,S*)-[^{11}C]MeNER labels regions of the human brain that are known to possess NET [2,3,5,8,15,19,24]. However, the non-specific binding of this ^{11}C-labeled compound appears rather high, which might limit the usefulness of it as a radioligand for NET *in vitro*, in contrary to what was reported by Wilson [29]. This difference might be associated with the thicker brain slices (100 μm vs. 20 μm) used for *in vitro* human brain autoradiography. The species difference of the tissues (rat vs. human) may also have importance for the non-specific binding. Further studies with tritium-labeled (*S,S*)-MeNER, will clarify its potential as an *in vitro* radioligand.

4.3. Positron emission tomography

A comparison of time-radioactivity curves, derived from injection of each of the enantiomers of [^{11}C]MeNER, dem-

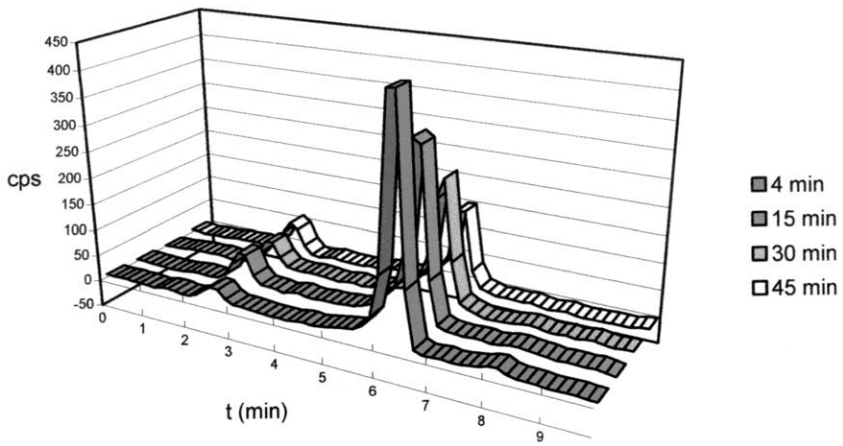

Fig. 7. Reversed phase HPLC radiochromatograms of samples take from monkey plasma at 4, 15, 30, 45 min after i.v. injection of [^{11}C]MeNER.

onstrated higher specific binding of the (*S,S*)-enantiomer. This was quite expected as the *in vitro* affinity of (*S,S*)-reboxetine is about 20 times higher than its (*R,R*)-enantiomer (3.6 nM vs. 85 nM) [32]. The regional distribution of [^{11}C]MeNER was in accordance with the known high density of NETs in the lower brainstem, mesencephalon and negligible density in the striatum [2,3,5,8,15,19,24]. The higher brain uptake of (*S,S*)-[^{11}C]MeNER into brain during the pretreatment experiment presumably arises from higher plasma levels of the radioligand due to displacement from peripheral NETs (from lungs etc.). Differences in total brain uptake were corrected for by relating binding in the ROI's to the striatum (a reference region for free radioligand concentration and non-specific binding in brain). Specific (*S,S*)-[^{11}C]MeNER binding in NET-rich regions to the striatum was reduced in the pretreatment experiment in a single monkey. This observation supports that a fraction of the binding represents specific binding to NET. Further characterization of specific (*S,S*)-[^{11}C]MeNER binding in monkey requires pretreatment experiments with the DAT selective compound, GBR12909, and the selective SERT inhibitor, citalopram. However, while this work was in progress, Wilson et al. reported that the *in vivo* binding of (*S,S*)-[^{11}C]MeNER in rats was insensitive to the inhibition of DATs and SERTs [29]. A drawback of using a mixture of Ketamine and Xylazine for inducing and maintaining anesthesia is that ketamine has been reported to bind to NET [13,26]; such binding might attenuate the PET signal by reducing binding potentials in the baseline experiment. However, the anesthetic dose was the same in both the baseline and the pretreatment experiment and should thus not challenge the conclusion that DMI has effect on (*S,S*)-[^{11}C]MeNER binding to NET.

4.4. Plasma metabolite studies

Labeled metabolites found in plasma after i.v. injection of [^{11}C]MeNER into monkey were less lipohilic than the parent radioligand. The metabolites are thus unlikely to pass the blood brain barrier and contribute to brain radioactivity.

5. Conclusions

[^{11}C]MeNER was labeled in high radiochemical yield and high specific radioactivity. (*S,S*)-[^{11}C]MeNER binding to NET in the brainstem was demonstrated *in vitro* using autoradiography on whole hemisphere cryosections from human post-mortem brains and *in vivo* by PET in Cynomolgus monkeys. (*S,S*)-[^{11}C]MeNER is a promising lead compound in the search for a PET radioligand for quantitation of NET in the human brain *in vivo.*

Acknowledgments

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